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GENETICS AND MOLECULAR BIOLOGY

F4- and F18-Positive Enterotoxigenic *Escherichia coli* Isolates from Diarrhea of Postweaning Pigs: Genomic Characterization

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ABSTRACT This study aimed to characterize *in silico* enterotoxigenic *Escherichia coli* F4- and F18-positive isolates ($n = 90$) causing swine postweaning diarrhea, including pathogenic potential, phylogenetic relationship, antimicrobial and biocide resistance, prophage content, and metal tolerance rates. F4 strains belonged mostly to the O149 and O6 serogroups and ST100 and ST48 sequence types (STs). F18 strains were mainly assigned to the O8 and O147 serogroups and ST10, ST23, and ST42. The highest rates of antimicrobial resistance were found against streptomycin, sulfamethoxazole, tetracycline, trimethoprim, and ampicillin. No resistance was found toward ciprofloxacin, cefotaxime, ceftiofur, and colistin. Genes conferring tolerance to copper (showing the highest diversity), cadmium, silver, and zinc were predicted in all genomes. Enterotoxin genes (*ltcA*, 100% F4, 62% F18; *astA*, 100% F4, 38.1% F18; *sta*, 18.8% F4, 38.1% F18; *stb*, 100% F4, 76.2% F18) and fimbria-encoding genes typed as F4ac and F18ac were detected in all strains, in addition to up to 16 other virulence genes in individual strains. Phage analysis predicted between 7 and 20 different prophage regions in each strain. A highly diverse variety of plasmids was found; IncFII, IncFIB, and IncFIC were prevalent among F4 isolates, while IncI1 and IncX1 were dominant among F18 strains. Interestingly, F4 isolates from the early 1990s belonged to the same clonal group detected for most of the F4 strains from 2018 to 2019 (ONT:H10-A-ST100-CH27-0). The small number of single-nucleotide polymorphism differences between the oldest and recent F4 ST100 isolates suggests a relatively stable genome. Overall, the isolates analyzed in this study showed remarkably different genetic traits depending on the fimbria type.

IMPORTANCE Diarrhea in the postweaning period due to enterotoxigenic *E. coli* (ETEC) is an economically relevant disease in pig production worldwide. In Denmark, prevention is mainly achieved by zinc oxide administration (to be discontinued by 2022). In addition, a breeding program has been implemented that aims to reduce the prevalence of this illness. Treatment with antimicrobials contributes to the problem of antimicrobial resistance (AMR) development. As a novelty, this study aims to deeply understand the genetic population structure and variation among diarrhea-associated isolates by whole-genome sequencing characterization. ST100-F4ac is the dominant clonal group circulating in Danish herds and showed high similarity to ETEC ST100 isolates from China, the United States, and Spain. High rates of AMR and high diversity of virulence genes were detected. The characterization of diarrhea-related ETEC is important for understanding the disease epidemiology and pathogenesis and for implementation of new strategies aiming to reduce the impact of the disease in pig production.

KEYWORDS pigs, diarrhea, enterotoxigenic *E. coli*, whole-genome sequencing, bioinformatics, enterotoxigenic, *Escherichia coli*

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Postweaning diarrhea (PWD) affects pigs after weaning, leading to significant economic costs for the pig industry due to weight loss and mortality as well as the cost of prevention (i.e., vaccination), treatment, and handling (1, 2). In addition to sudden death or profuse diarrhea, the disease is accompanied by growth retardation in surviving piglets (1, 3). During acute outbreaks, mortality due to PWD may reach 20 to 30% over a 1 to 2 months' time span among infected pigs (3). As it is one of the most common reasons for the use of antimicrobials in the pig industry worldwide, PWD significantly contributes to the problem of antimicrobial resistance (AMR) development (2, 4, 5).

Enterotoxigenic *Escherichia coli* (ETEC) is the main etiological agent involved in PWD worldwide. The ETEC pathotypes in pigs are characterized by the expression of specific fimbrial adhesins, which mediate bacterial colonization of the gut mucosal surface. The most commonly detected types of fimbriae are F4 (previously termed K88) and F18 (F107, 2134P, and 8813). Both include different antigenic variants, three for F4 (ab, ac, and ad), with F4ac being the most prevalent, and two types for F18 (ab and ac), with F18ac being the main one associated with PWD (6, 7). Intestinal adhesion and subsequent colonization by ETEC depends on F4- or F18-specific receptors, the presence of which is essential for ETEC to cause disease (8). ETEC F4 is usually related to PWD of recently weaned piglets occurring 2 to 3 days after weaning (classical PWD), while F18 is commonly found associated with diarrhea 2 to 6 weeks after weaning. The age-dependent expression of F4 and F18 receptors in the small intestine might explain why ETEC F4 infection mainly takes place right after weaning as well as during the neonatal period, while ETEC F18 infection mainly occurs later in the postweaning period (5).

Once ETEC bacteria have adhered and colonized the small intestine, they can produce an enterotoxin(s) leading to diarrhea. Both ETEC F4 and F18 are reported to encode two classes of enterotoxins, heat-labile (LT) and heat-stable (Sta, Stb, and EAST1, for enteroaggregative heat-stable toxin 1) enterotoxins, which induce secretory diarrhea in the pigs (1, 2, 9–11). The predominant serogroup of ETEC associated with classical PWD in pigs worldwide is O149-F4 (1).

In Denmark and other countries, zinc oxide (ZnO) in therapeutic concentrations has been used in recent decades to prevent PWD in the first 14 days after weaning. ZnO has been found to improve growth performance and reduce scours (ETEC induced) in weaning piglets (12). Moreover, ZnO reduces bacterial adhesion and inflammatory cytokine expression and prevents the disruption of membrane integrity caused by ETEC (13). However, due to the environmental toxicity and potential coselection for AMR, the use of ZnO will be banned in pig production in the EU by 2022 (https://www.ema.europa.eu/en/medicines/veterinary/referrals/zinc-oxide). Other strategies that have been used to tackle PWD caused by ETEC include a breeding program (DanBred), which has been implemented in some Danish farms since 2003, and vaccines. The former consists of breeding pigs that do not express the F4ac-specific receptors on the intestinal mucosa, aiming at reducing the occurrence of diarrhea due to ETEC F4 (5). The live attenuated vaccine Coliprotec F4/F18 (https://www.ema.europa.eu/en/medicines/veterinary/EPAR/coliprotec-f4f18) has been developed to diminish the incidence of PWD caused by both ETEC F4 and F18 bacteria. Besides these preventive strategies, neomycin, apramycin, spectinomycin, tetracycline, amoxicillin, and sulfadiazine-trimethoprim are the antimicrobials commonly used to treat PWD in Denmark (14).

In the present work, we characterized a collection of Danish ETEC F4- and F18-positive strains through whole-genome sequencing (WGS) to analyze the pathogenic potential of the strains through the identification of relevant virulence factors and to determine the occurrence of AMR as well as biocide and metal resistances. Further, the analysis allowed us to understand the population genetic structure and variation among strains that are associated with PWD. Moreover, we also analyzed the phylogenetic relationship between the Danish strains under study and swine ETEC strains from other countries of the world.

TABLE 1 Serotype, phylogroup, sequence type, and clonotypes associated with fimbrial antigens in the 90 ETEC isolates from pigs

Clonal group (serotype-PG-ST-CH)[a]	No. (%) of isolates	Fimbrial antigen
O149:H10-A-ST100-CH27-0	49 (54.4)	F4
ONT:H10-A-ST100-CH27-0	6 (6.7)	F4
O149:HNT-A-ST100-CH27-0	1 (1.1)	F4
O6:H16-A-ST48-CH11-34	11 (12.2)	F4
O8:H19-C-ST90-CH4-54	2 (2.2)	F4
O8:H17-C-ST23-CH4-54	6 (6.7)	F18
ONT:H4-A-ST10-CH11-24	5 (5.6)	F18
O141:H4-A-ST10-CH11-24	2 (2.2)	F18
O147:H14-D-ST42-CH28-65	3 (3.3)	F18
ONT:H14-D-ST42-CH28-65	2 (2.2)	F18
O29:H12-B1-ST155-CH4-121	1 (1.1)	F18
ONT:H19-C-ST90-CH4-0	1 (1.1)	F18
O8:H31-E-ST3524-CH23-31	1 (1.1)	F18

[a]PG, phylogroup; ST, sequence type; CH, clonotype.

RESULTS

Phylogroups, sequence types, clonotypes, and serotypes. Most of the ETEC isolates belonged to phylogroup A (74 isolates, 82.2%), and the remaining were assigned to four different phylogroups, B1 (one isolate, 1.1%), C (nine isolates, 10%), D (five isolates, 5.5%), and E (one isolate, 1.1%). The strains displayed eight different sequence types (STs) by multilocus sequence typing (MLST) (ST10, ST23, ST42, ST48, ST90, ST100, ST155, and ST3524), with ST100 accounting for 56 (62.2%) isolates. Based on *fumC-fimH* allele combinations, eight clonotypes (CHs) were identified, with CH27-0 being the most prevalent type (56 isolates, 62.2%), corresponding to strains assigned to ST100 (see Table S2 in the supplemental material).

The SerotypeFinder tool detected six O serogroups (O6, O8, O29, O141, O147, and O149) and eight different H antigens (H4, H10, H12, H14, H16, H17, H19, and H30). It was not able to predict the O and H antigen in 14 and 8 isolates, respectively. Overall, 13 O:H combinations (serotypes) were found (Table S2), with O149:H10 being the most common serotype identified (49 isolates, 54.4%), followed by O6:H16 (11 isolates, 12.2%).

The association between clonal groups (defined by serotype, phylogroup, sequence type, and clonotype) and fimbrial type detected among the strains is shown in Table 1. Two main clonal groups were identified among the 69 F4-positive isolates, O149/ONT:H10/HNT-A-ST100-CH27-0 and O6:H16-A-ST48-CH11-34, representing 81.2% (56 strains) and 15.9% (11 strains) of the isolates, respectively. The remaining F4-positive isolates belonged to the O8:H19-C-ST90-CH4-54 clonal group (two isolates, 2.9%). Interestingly, five out of the six F4-positive isolates isolated in the early 1990s also belonged to the ONT:H10-A-ST100-CH27-0 clonal group shown for most of the F4 isolates collected during the period of 2018 to 2019 (Table S2). In contrast to the homogeneity observed among F4 isolates, the 21 F18-positive isolates showed a higher diversity and were assigned to six different clonal groups, of which O141/ONT:H4-A-ST10-CH11-24 (33.3%; seven isolates) and O8:H17-C-ST23-CH4-54 (28.6%; six isolates) were the predominant ones. The remaining F18-positive isolates were assigned to the O147/ONT:H14-D-ST42-CH-28-65 (five isolates, 23.8%), O29:H12-B1-ST155-CH4-121, ONT:H19-C-ST90-CH4-0 (*E. coli* Nysø), and O8:H31-E-ST3524-CH23-31 (one isolate each, 4.7%) clonal groups. F18-positive strains from the same herd belonged to the same clonal group, including seven, six, and five isolates from herd D, herd B, and herd C, respectively (Table S2).

Phylogeny analysis of ETEC isolates. The raw read mapping of all 90 genomes to the reference *E. coli* K-12 genome showed that 3,475,685 out of 4,641,652 (74.8%) nucleotide positions in the reference genome were present in all of the analyzed genomes. A total of 42,172 variable nucleotide positions were detected in this core genome (Table S3).

Isolates were clustered into four major clades (I, II, III, and IV) and grouped according to their ST (Fig. 1). Clade I included seven ST10-F18 isolates recovered from the same

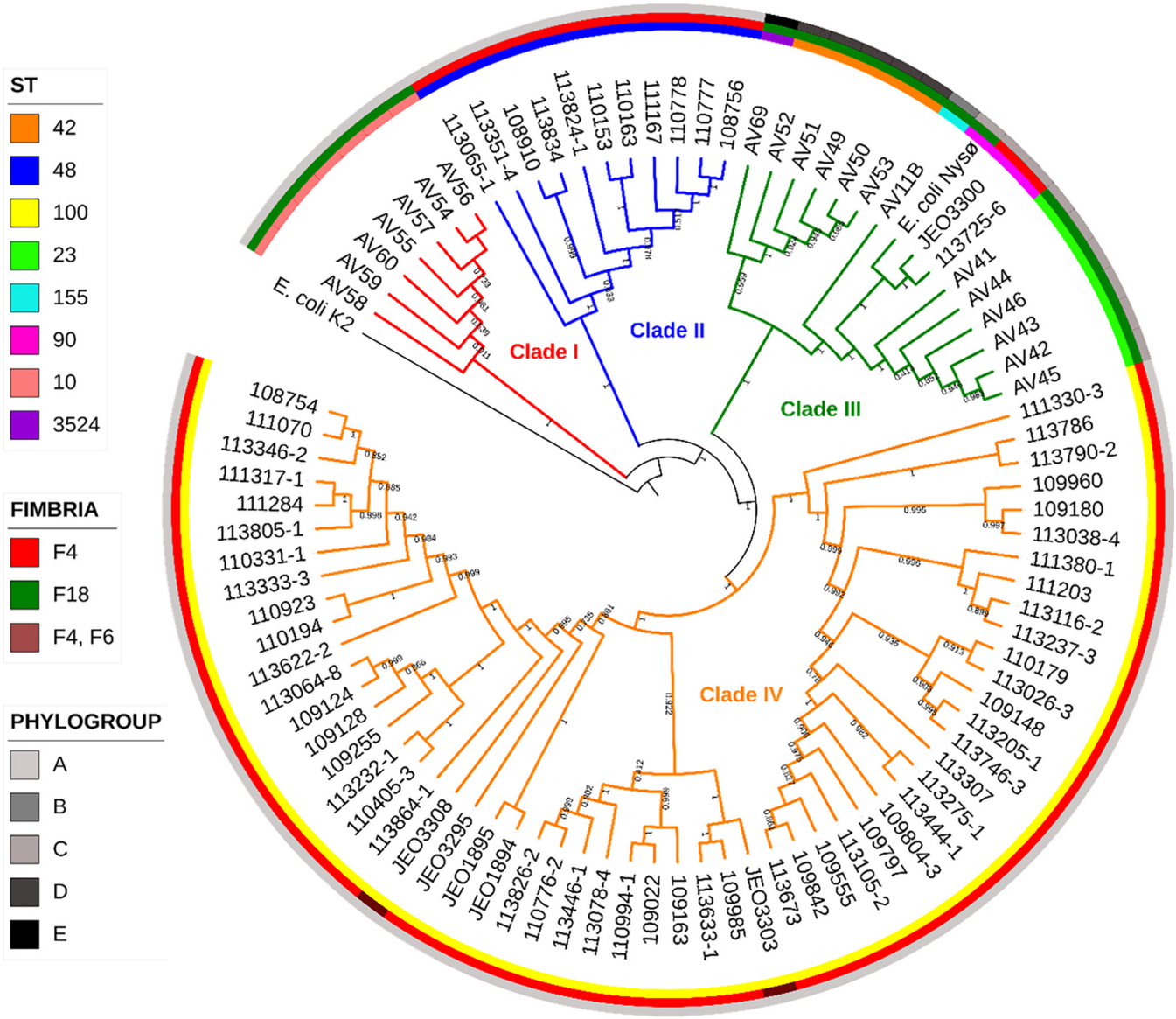

FIG 1 SNP-based phylogeny of the 90 ETEC isolates from pigs. Colors in the outer ring correspond to phylogroups, the middle ring to fimbria type, and the inner ring to sequence type.

herd and showed 1 to 14 single-nucleotide polymorphism (SNP) differences. Isolates belonging to ST48 and encoding F4 fimbriae (11 isolates) were grouped in clade II and showed from 6 (between isolates from the same herd) to 633 SNP differences. Clade III encompassed isolates assigned to different STs (ST155, ST23, ST42, ST3524, and ST90), and all except two ST90 isolates were F18 positive. This cluster was divided into two well-defined subclades: subclade A (including five ST42 isolates from the same herd, with numbers of SNP differences ranging from 4 to 18, and one ST3524 isolate) and subclade B, containing six ST23 isolates (between 3 and 21 SNP differences), three ST90 (two of them were F4 positive) strains (between 104 and 556 SNP differences), and a single ST155 isolate. Lastly, clade IV consisted of F4-positive isolates belonging to ST100 (56 isolates) and was split into two subclades, with the number of different SNPs spanning from 16 to 866 among all genomes and from 4 to 15 among isolates belonging to the same herd. Numbers of SNP differences among the five F4-ST100 isolates from the 1990s and F4-ST100 strains recovered during 2018 to 2019 ranged from 130 to 699.

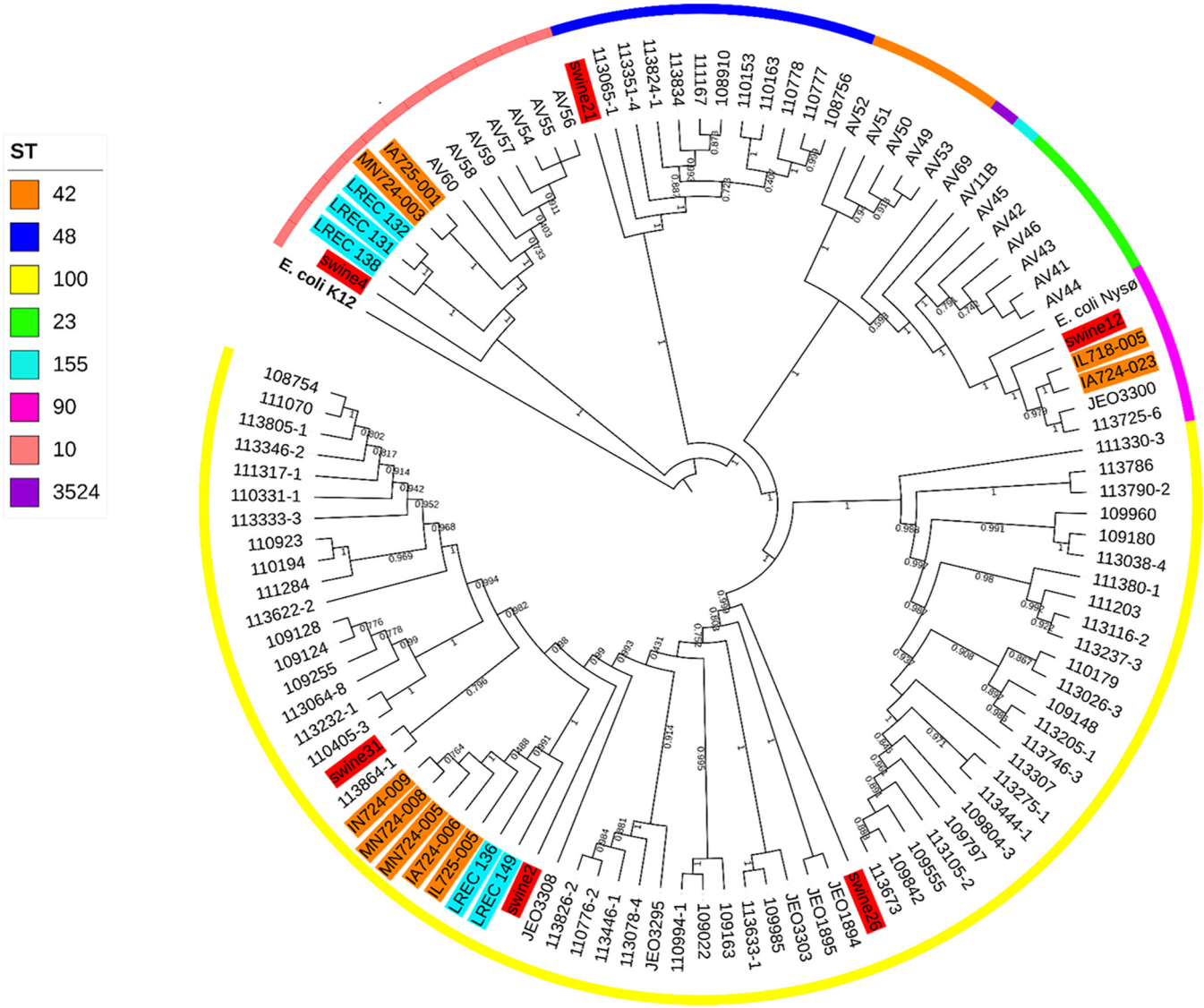

FIG 2 SNP-based phylogeny of the 90 ETEC isolates from pigs in Denmark and the 20 porcine ETEC isolates from different countries. Colors in the ring correspond to sequence type. Isolates highlighted in red, orange, and blue correspond to isolates from China, the United States, and Spain, respectively.

We investigated the relationship between our strains and porcine ETEC isolates from other countries (Fig. 2 and Table S11). Phylogenetic analysis based on SNP indicated that the Danish ST100 isolates are closely related to ST100 isolates from China (between 120 and 443 SNP differences), Spain (178 to 708), and the United States (196 to 466). The lowest numbers of SNP differences among ST10 isolates were detected versus ST10 strains from the United States (300 and 301), followed by Spain (1,779 to 1,852) and China (2,023 to 2,025). Regarding ST48, the single isolate from China, included for comparisons, showed between 4,163 and 4,291 SNP differences compared to the Danish ST48 isolates. Finally, our ST90 strains were more similar to the ST90 isolate from China (between 80 and 268 SNP differences) than to the ST90 strains from the United States (ranging from 388 to 476 SNP differences).

Antimicrobial resistance phenotypes and genotypes. The antimicrobial susceptibility testing revealed that 85 (94.4%) of the isolates were resistant to at least one of the antimicrobials investigated, 60 (66.7%) were multidrug resistant (MDR), and only five were susceptible to all antimicrobials. High rates of resistance were found against streptomycin (68.9% of the isolates), sulfamethoxazole (67.8%), tetracycline (56.7%),

TABLE 2 Prevalence of antimicrobial resistance among the 90 ETEC isolates from pigs

Antimicrobial[a]	No. (%) of resistant isolates	No. (%) of isolates of each fimbrial type[b]		*P* value	OR[c] (95% CI)
		F4	F18		
Ampicillin	47 (48.3)	35 (50.7)	12 (57.1)	0.6282	
Amoxicillin-clavulanic acid	1 (1.1)	1 (1.5)	0	>0.9999	
Chloramphenicol-florfenicol	15 (16.7)	9 (13.4)	6 (28.5)	0.1064	
Apramycin	8 (8.9)	7 (10.14)	1 (4.7)	0.6752	
Gentamicin	6 (6.7)	8 (11.6)	1 (4.7)	0.6792	
Neomycin	23 (25.6)	16 (23.2)	7 (33.3)	0.3964	
Spectinomycin	50 (55.6)	35 (50.5)	15 (71.42)	0.1327	
Streptomycin	62 (68.9)	**53 (76.8)**	9 (42.9)	0.0061	4.417 (1.491–12.48)
Sulfamethoxazole	61 (67.8)	46 (66.7)	15 (71.4)	0.7932	
Tetracycline	51 (56.7)	42 (60.9)	9 (42.8)	0.2084	
Trimethoprim	48 (53.3)	35 (50.7)	13 (62)	0.4568	
Nalidixic acid	8 (8.9)	8 (11.6)	0	0.1901	

[a]The breakpoints used correspond to EUCAST epidemiological cutoff values (ECOFFs) for ampicillin (8 μg/ml), chloramphenicol-florfenicol (8 μg/ml), gentamicin (2 μg/ml), nalidixic acid (8 μg/ml), neomycin (8 μg/ml), spectinomycin (64 μg/ml), streptomycin (16 μg/ml), sulfamethoxazole (64 μg/ml), tetracycline (8 μg/ml), and trimethoprim (2 μg/ml). EUCAST clinical breakpoints for amoxicillin-clavulanic acid ($R > 8$ μg/ml) and DANMAP 2015 for apramycin ($R > 32$ μg/ml) were also used.
[b]Percentage is estimated based on the total number of strains associated with each fimbrial type. Significant differences ($P < 0.05$) are indicated in boldface.
[c]Odds ratio (OR) is indicated when the *P* value is <0.05; CI, confidence interval.

spectinomycin (55.6%), trimethoprim (53.3%), and ampicillin (48.3%). Importantly, none of the isolates was resistant to ceftiofur, cefotaxime, colistin, or ciprofloxacin, which are considered highly critical drugs in human medicine (15) (Table 2). In addition, one and eight F4-positive isolates showed resistance to amoxicillin-clavulanic acid and nalidixic acid, respectively, while none of the F18 isolates tested positive for these drugs (Table 2).

Results from kappa statistical analysis showed that there was an almost perfect agreement between phenotypic resistance and the *in silico* prediction of resistance genotype (Table 3), which identified a total of 39 different AMR genes (Table S2). The genes bla_{TEM-1B}, *tet*(A), and *dfrA1* were the most commonly detected among ampicillin-, tetracycline-, and trimethoprim-resistant isolates, respectively. Similarly, *sul1* and *sul2* were the predominant genes responsible for sulfonamide resistance. A total of 16 different genes encoding aminoglycoside-modifying enzymes were identified, with the *aph* [phosphotransferases, i.e., *aph(3′)-Ia*, *aph(3′)-Ib*, *aph(3″)-Ib*, *aph(4)-Ia*, and *aph(6)-Id*] and *aadA* (nucleotidyltranferases, i.e., *aadA1*, *aadA2*, *aadA5*, *aadA11*, *aadA12*, *aadA17*, *aadA22*, and *aadA24*) genes being the most common, consistent with the high resistance to spectinomycin and streptomycin, respectively. Regarding phenicols, *catA1*, *cmlA1*, and *floR* genes were found among the resistant isolates (Table 3 and Table S2). Susceptibility to macrolides and lincosamides was not phenotypically tested; however, genes conferring resistance to both classes of drugs were detected (Table 3 and Table S2).

Notably, none of the isolates was found to harbor genes that suggested extended-spectrum beta-lactamase (ESBL) production or transferrable colistin resistance (*mcr*-class genes).

The ResFinder bioinformatics tool also allows the identification of chromosomal mutations related to antimicrobial resistance. Eight isolates, phenotypically resistant to nalidixic acid, showed a single chromosomal mutation in the *gyrA*(S83L) gene. The substitution V161G, associated with colistin resistance, was also detected in the *pmrB* gene in five isolates; however, these isolates were phenotypically susceptible to the antimicrobial. In addition, one isolate had a nucleotide change in the *ampC* promoter (32T→A) that is associated with resistance to beta-lactams (Table S2).

Prediction of biocide and metal tolerance genes. The ETEC isolates showed a large variety of biocide resistance and metal tolerance genes (Fig. 3 and Table S4). A total of 117 different genes were detected, and the number of genes per isolate ranged from 40 to 88. Eleven genes associated with metal tolerance and/or related to metal transport and metabolism as well as biocide resistance were shared by all

TABLE 3 Antimicrobial resistance genes detected among the 90 ETEC isolates from pigs

Antimicrobial group and gene	Total no. (%) of isolates	No. (%) of isolates of each fimbrial type[a]		Kappa	*P* value[b]
		F4	F18		
Beta-lactams					
bla_{TEM-1A}	4 (4.4)	4 (5.8)	0	0.933	0.0001
bla_{TEM-1B}	42 (46.7)	30 (43.5)	12 (57.1)		
bla_{TEM-30}	1 (1.1)	1 (1.4)	0		
Aminoglycosides					
aph (phosphotransferases)	58 (64.4)	50 (72.5)	8 (38.1)	0.927	0.0001
aadA (nucleotidyltransferases)	57 (63.3)	42 (60.9)	15 (71.4)		
aac (acetyltransferases)	9 (10)	8 (11.6)	1 (4.8)		
Phenicols					
catA1	3 (3.3)	3 (4.3)	0	1.000	0.0001
cmlA1	8 (8.9)	2 (2.9)	6 (28.6)		
floR	5 (5.6)	5 (7.2)	0		
Macrolides					
mdf(A)	90 (100)	69 (100)	21 (100)	ND	ND
mph(A)	8 (8.9)	8 (11.6)	0		
mph(B)	7 (7.8)	7 (10.1)	0		
erm(B)	9 (10)	9 (13)	0		
Lincosamides					
lnu(F)	5 (5.6)	5 (7.2)	0	ND	ND
lnu(G)	5 (5.6)	5 (7.2)	0		
Sulfonamides					
sul1	30 (33.3)	28 (40.6)	2 (9.5)	0.898	0.0001
sul2	42 (46.7)	35 (50.7)	7 (33.3)		
sul3	9 (10)	3 (4.3)	6 (28.6)		
Tetracycline					
tet(A)	40 (44.4)	32 (46.4)	8 (38.1)	1.000	0.0001
tet(B)	13 (14.4)	12 (17.4)	1 (4.8)		
tet(X)	1 (1.1)	1 (1.4)	0		
Trimethoprim					
dfrA1	34 (37.8)	27 (39.1)	7 (33.3)	0.978	0.0001
dfrA5	2 (2.2)	2 (2.9)	0		
dfrA12	8 (8.9)	2 (2.9)	6 (28.6)		
dfrA14	5 (5.6)	5 (7.2)	0		
dfrA17	2 (2.2)	2 (2.9)	0		

[a]Percentage is estimated based on the total number of strains associated with each fimbrial type.
[b]A *P* value of <0.05 is considered significant.

of the strains in the collection: *corA* (magnesium [Mg], cobalt [Co], nickel [Ni], and manganese [Mn]), *glpF* (antimony [Sb] and arsenic [As]), *mgtA* (Co and Mg), *zntR* (*yhdM*) (zinc [Zn]), *phoB* (benzalkonium chloride and chlorhexidine), *mdtF* (*yhiV*), *mdtK* (*ydhE*) (several biocides, including benzalkonium chloride and ethidium bromide), *ostA* (*lptD*) (N-hexane), *emrB* (phenylmercury acetate, 2-chlorophenyl-hydrazine, and carbonylcyanide m-chlorophenyl hydrazine), and *acrB* and *acrF* (*envD*) (acriflavine). In addition, different genes conferring metal tolerance to cadmium (Cd), silver (Ag), mercury (Hg), lead (Pb), copper (Cu), and tellurium (Te) were predicted in each strain.

The highest diversity of genes conferring biocide resistance and metal tolerance were identified for copper (16 genes), hydrogen peroxide (15 genes), ethidium bromide (13 genes), sodium deoxycholate (13 genes), cadmium (12 genes), acriflavine (11 genes), and zinc (11 genes).

Genes involved in the uptake and transport of metals are ubiquitously found in bacteria, since metal ions play a role in many different biological processes and are

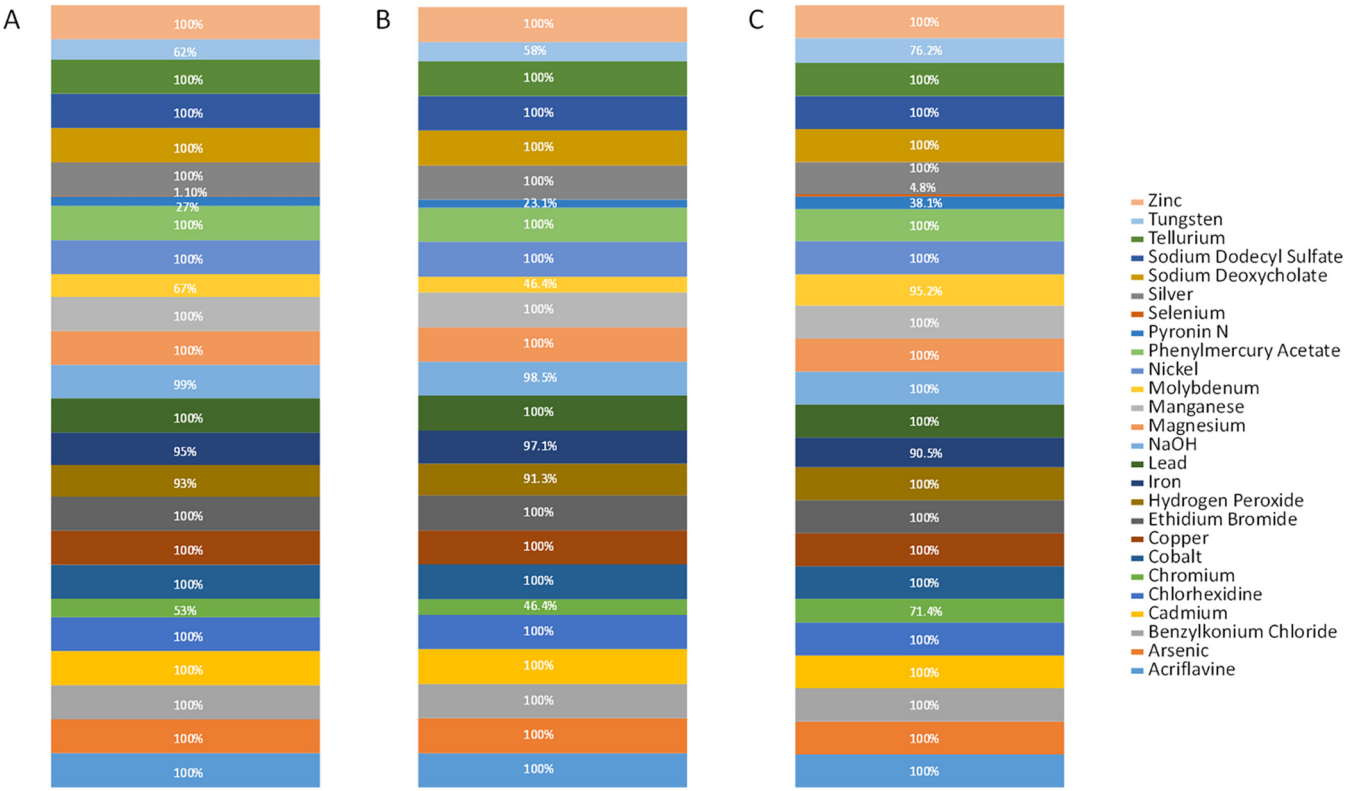

FIG 3 Distribution of metal tolerance and biocide resistance among the 90 ETEC isolates from pigs (A), the F4-positive isolates (B), and the F18-positive isolates (C).

essential for bacterial survival (16). All genes detected in the ETEC isolates were predicted in *E. coli* K-12, with the exception of those presumably conferring metal tolerance to Co (*cuiD*, *pcoB*, and *pcoS*), Hg (*merA*, *merE*, and *merR*), quaternary ammonium compounds (*qacE*, *qacEdelta1*, and *qacF*), Ag (*silA*, *silE*, and *silP*), and Te (*terB*, *terC*, *terD*, *terW*, and *terZ*), which were exclusively predicted in ETEC strains (data not shown).

Virulence genes carried by ETEC isolates. The VirulenceFinder bioinformatics tool corroborated the presence of F4 and F18 fimbrial genes in 69 and 21 *E. coli* isolates, respectively, and further identified them as F4ac (100%) and F18ac (100%). Two isolates also were predicted to carry F6 type fimbria together with F4. WGS analysis also confirmed the presence of *sta* (21 isolates, 23.3%) and *stb* (85 isolates, 94.4%) heat-stable enterotoxins in those isolates positive for the toxins by PCR.

Apart from the fimbrial F4 and F18 and enterotoxin *sta* and *stb* genes, a total of 16 different virulence genes were predicted among the 90 *E. coli* isolates, such as *ltcA*, the gene associated with the heat-labile enterotoxin (LT) and present in most of the isolates (82 isolates, 91.1%). The gene *astA* (encoding the EAST-1 heat-stable toxin) and the gene *iha* (encoding a nonhemagglutinating adhesion protein [17]) were also present in 85% of the isolates (Table 4). All isolates carried at least five different virulence genes, and 60% of the isolates harbored eight or more virulence determinants.

Notably, statistically significant differences were detected in the distribution of virulence genes depending on the fimbria type (Table 4). While all F4-positive isolates harbored *astA*, *ltcA*, and *stb* genes, only a portion of F18-positive strains carried these genes (38%, 61%, and 76%, respectively). The genes *gad* (encoding a glutamate decarboxylase, enabling resistance to gastric acidity) (18) (35 isolates), *capU* (hexosyltransferase) (54 isolates), and the *cba* (33 isolates), *cma* (42 isolates), and *celB* (eight isolates) colicin-encoding genes (bacteriocins active against closely related *E. coli* bacteria or other members of the *Enterobacteriaceae*) (19) also were more frequently detected among F4- than among F18-positive strains. However, the genes *lpfA* (long

TABLE 4 Distribution of virulence genes (other than F4- and F18-encoding fimbria) among the 90 ETEC isolates

Function and gene	Total no. (%) of isolates	No. (%) of isolates of each fimbrial type[a]		*P* value	OR[b] (95% CI)
		F4	F18		
Toxins					
astA	77 (85.6)	**69 (100)**	8 (38.1)	<0.0001	∞ (25.71–∞)
ltcA	82 (91.1)	**69 (100)**	13 (62)	<0.0001	∞ (9.402–∞
sta	21 (23.3)	13 (18.8)	8 (38.1)	0.0820	
stb	85 (94.4)	**69 (100)**	16 (76.2)	0.0005	∞ (5.465–∞)
Fimbriae					
lpfA	15 (16.7)	2 (2.9)	**13 (61.9)**	<0.0001	0.01837 (0.003635–0.08)
fasA	2 (2.2)	2 (2.9)	0	>0.9999	
Adhesion					
iha	77 (85.6)	57 (82.6)	20 (95.2)	0.2854	
air	4 (4.4)	0	**4 (19)**	0.0023	0 (0.0–0.02885)
Colicin					
cba	33 (36.7)	**33 (47.8)**	0	<0.0001	∞ (4.748–∞)
cma	47 (52.2)	**42 (60.9)**	5 (23.8)	0.0053	4.978 (1.631–13.29)
celB	10 (11.1)	8 (11.6)	2 (9.5)	>0.9999	
Microcin					
mchB	9 (10)	9 (13)	0	0.1096	
mchC	9 (10)	9 (13)	0	0.1096	
mchF	9 (10)	9 (13)	0	0.1096	
mcmA	9 (10)	9 (13)	0	0.1096	
Others					
capU	55 (61.1)	**54 (78.3)**	1 (4.8)	<0.0001	72 (11.75–759.7)
gad	40 (44.4)	**35 (50.7)**	5 (23.8)	0.0439	3.294 (1.09–8.811)
sepA	10 (11.1)	10 (14.5)	0	0.1088	
iss	20 (22.2)	1 (1.4)	**19 (90.5**)	<0.0001	0.001548 (0.0001506–0.0)
eilA	5 (5.6)	0	**5 (23.8)**	0.0005	0 (0.0–0.1830)

[a]Percentage is estimated based on the total number of strains associated with each fimbria type. Significant differences ($P < 0.05$) are indicated in boldface.
[b]The odds ratio (OR) is indicated when the *P* value is <0.05; CI, confidence interval; ∞, infinity.

polar fimbriae) (13 isolates) and *iss* (increased serum survival; role in extraintestinal pathogenic *E. coli* [ExPEC] virulence [20]) (19 isolates) were predominantly found in F18-positive isolates. In addition, microcin-encoding genes (nine isolates) and *sepA* (*Shigella* extracellular protein A, involved in tissue invasion) (10 isolates) (21) were exclusively identified in F4-positive strains, while *air* (enteroaggregative immunoglobulin protein with epithelial adhesion function) (four isolates) and *eilA* (*Salmonella* HilA homolog; transcriptional activator of SPI-1 genes) (five isolates) (22) were unique to F18-positive isolates. Hybrid variants VTEC (verotoxin-producing *E. coli*)/ETEC were not identified in the present work.

***In silico* plasmid detection and prediction of cooccurrence of AMR genes with virulence and metal tolerance genes.** Twenty-one different plasmid replicons were predicted among the *E. coli* strains. The most prevalent replicon types were IncFII (92.2% of the strains) and IncFIB (91.1%), followed by IncI1 (72.2%), IncFIC (54.4%), and IncX1 (36.7%), and the number of plasmid replicons per isolate ranged from three to eight (Table 5). At least one IncF replicon was detected in all the isolates.

With regard to fimbrial type, all F4-positive isolates contained replicons of both IncFII and IncFIB types, while all F18-positive strains harbored replicons of the IncX1 type and all but one carried IncI1 (95.2%). The latter was found in 65.2% of the F4-positive strains, and the IncI2 type was exclusively detected in F4 isolates. The replicon IncX1 also was found in all F18 isolates and only in 12 F4 isolates, while the replicon IncX4 was unique to three F4 isolates. In addition, IncHI, IncN, and IncQ replicons were only found in F4-positive isolates (Table 5).

According to the plasmidSPAdes and ResFinder bioinformatics tools, the majority of the AMR genes detected in the ETEC genomes were predicted to be plasmid located.

TABLE 5 Plasmid replicons predicted by PlasmidFinder among the 90 ETEC isolates

Plasmid group and replicon	Total no. (%) of isolates	No. (%) of each fimbrial type[a]		*P* value	OR[b] (95% CI)
		F4	F18		
IncF					
FII	83 (92.2)	**69 (100)**	14 (66.7)	<0.0001	∞ (7.269–∞)
FIB	82 (91.1)	**69 (100)**	13 (61.9)	<0.0001	∞ (9.402–∞)
FIC	49 (54.4)	41 (59.4)	8 (38.1)	0.1323	
FIA	1 (1.1)	1 (1.4)	0	>0.9999	
IncI					
I1	65 (72.2)	45 (65.2)	**20 (95.2**)	0.0057	0.09375 (0.0086–0.6311)
I2	12 (13.3)	12 (17.4)	0	0.0614	
IncX					
X1	33 (36.7)	12 (17.4)	**21 (100)**	<0.0001	0.000 (0.000–0.06711)
X4	3 (3.3)	3 (4.3)	0	>0.9999	
IncQ					
Q1	21 (23.3)	**21 (30.4)**	0	0.0024	∞ (2.202–∞)
Col-like					
Col156	15 (16.7)	13 (18.8)	2 (9.5)	0.5056	
Col4400II	2 (2.2)	2 (2.9)	0	>0.9999	
ColRNAI	2 (2.2)	2 (2.9)	0	>0.9999	
IncHI					
1A	1 (1.1)	1 (1.4)	0	>0.9999	
1B	1 (1.1)	1 (1.4)	0	>0.9999	
2	7 (7.7)	7 (10.1)	0	0.1933	
2A	7 (7.7)	7 (10.1)	0	0.1933	
IncN	12 (13.3)	12 (17.4)	0	0.0614	
IncB/O/Z	10 (11.1)	3 (4.3)	**7** (**33.3**)	0.0012	0.09091 (0.02421–0.415)
IncY	5 (5.5)	5 (7.2)	0	0.5869	
p0111	21 (23.3)	14 (20.3)	7 (33.3)	0.2450	
pKPC	3 (3.3)	3 (4.3)	0	>0.9999	

[a]Percentage is estimated based on the total number of strains associated with each fimbrial type. Significant differences ($P < 0.05$) are indicated in boldface.
[b]The odds ratio (OR) is indicated when the *P* value is <0.05; CI, confidence interval.

Specifically, bla_{TEM}, *floR*, *catA*, *cmlA*, and *sul3* genes were always detected in plasmid contigs in all isolates. On the contrary, *mdf*(A) was predicted to be a chromosomal gene, since it was not detected in any plasmid component (Table S5).

Similarly, toxins were predicted to be located on plasmids in most of the strains: *sta* (71.4%, 15 out of 21 positive isolates), *stb* (83.5%, 71 out of 85 positive isolates), *ltcA* (87.8%, 72 out of 82 positive isolates), and *astA* (89.6%, 69 out of 77 positive isolates). In 58 out of the 90 genomes, the genes *astA* and *stb* were found in the same contigs, predicted to be a plasmid, and 22 of these also contained the gene *ltcA* in the same plasmid contig. Both F4 and F18 fimbrial genes appeared to be plasmid associated in all genomes too (Table S6). Interestingly, where both AMR and virulence genes were predicted to be carried by plasmids in a strain, these two types of genes were identified in the same plasmid component but not in the same plasmid contig.

Results from BacMet bioinformatics analysis were analyzed together with the plasmid bioinformatics tool; *pitA* and *zintA* (*yodA*) genes, conferring Zn tolerance, were predicted to be plasmid located in 4 and 16 out of 32 and 65 ETEC isolates that harbored these genes. This analysis also predicted that genes related to Cu tolerance were putatively plasmid located in some cases: *cuiD* in one out of six strains, *cueO* in 1 out of 27 strains, *pcoS* in four out of six strains, and *pcoB* in 34 out of 53 strains. Despite all of these Zn and Cu tolerance genes being predicted in the same plasmid component as certain AMR genes, cooccurrence in the same plasmid contig was not detected (Table S7). Additionally, most of the *sil*-like genes, *mer*-like genes, and *ter*-like genes encoding tolerance to Ag, Hg, and Te, respectively, were also predicted to be plasmid

located and were also identified in the same plasmid component as several AMR genes (Table S8).

Prediction of prophage sequences in ETEC strains. The *in silico* analysis of the 90 ETEC genomes with PHASTER resulted in the prediction of a minimum of 7 to a maximum of 20 different prophage regions in each strain (Table S9). The genome sizes of these prophage regions spanned from 2.7 to 107.9 kb, with a GC content ranging from 40.45 to 57.46%. This is very similar to the GC content of *E. coli*, that is, approximately 50% (23). Of the total predicted prophages, 39.1% were intact, whereas 48.2% were incomplete and the remaining 12.6% questionable (Fig. S1). The genome size, the GC content, and the number of prophage regions identified did not statistically differ between F4 and F18 strains. Most of the prophage regions identified in the ETEC genomes showed similarity to P88 (GenBank accession no. NC_026014) and PhiP27 (NC_003356) *Enterobacteriaceae* prophages (from 3.9% to 93.3% and from 4.8% to 60.9%, respectively). P88 is an inducible prophage of *E. coli* strain K88, able to lyse avian pathogenic *E. coli* strains (24), and the coliphage PhiP27 is an Stx2e-encoding phage, but since none of the ETEC isolates encoded Shiga toxins and the highest similarity is only 60.9%, the ETEC prophages could be part of the gene pool producing PhiP27 by recombination (25, 26).

DISCUSSION

The characterization of ETEC causing PWD is important for understanding disease epidemiology and pathogenesis and for the implementation of new strategies aiming to reduce the impact of the disease in the pig industry. In this study, we characterized *in silico* a collection of 90 ETEC F4- and F18-positive isolates to determine the levels of antimicrobial and biocide resistance, metal tolerance, and virulence genes associated with PWD. The antimicrobial resistance phenotype was also investigated. We also determined how many clonal groups are circulating in the production system as well as the phylogenetic relationships between ETEC strains currently causing PWD in Denmark and in other countries worldwide.

Overall, ETEC isolates showed significantly different genetic traits depending on the fimbria type. It should be mentioned that while the 69 F4-positive strains were recovered from at least 30 different herds, the 21 F18-positive isolates were collected from just five herds.

ETEC isolates causing PWD are commonly reported to belong to the serogroups O8, O138, O139 (often associated with edema disease), O141, 147, O149, and O157 (1, 27, 28), of which O8, O141, O147, and O149 were predicted to be among strains in the current investigation, together with O29 and O6, both commonly recognized serogroups associated with ETEC and enteroinvasive *E. coli* (EIEC) in humans (29). Differences were observed concerning serogroups between F4- and F18-positive isolates. O149 was the predominant serogroup among F4 isolates (72.46% versus 0%, $P < 0.0001$), while O8 was the most prevalent among F18 isolates (2.9% versus 33.33%, $P < 0.0001$). O149 and O138 (not detected here) have been the most frequent serogroups related to the classical ETEC F4 and F18, respectively, in Denmark (28) and other countries (30–32). As reported in Australia, the United States, Canada, Germany, and Thailand (32, 33), ST100 was the most frequent ST among the Danish porcine ETEC isolates, and ST48 and ST10 were also commonly observed. The latter STs belong to the largest clonal complex, 10, within *E. coli*, with isolates from both animal and human sources acting as commensals or as pathogens, and generally are associated with antimicrobial susceptibility and low virulence (34). ST10 is dominant in Spain among *mcr*-positive isolates and in China (35–37), followed by ST48 in the latter country (37).

To study the genetic relatedness of the collected *E. coli* isolates, phylogenetic analysis based on SNPs was performed. As expected, the isolates clustered depending on their ST in four well-defined clades, with three of them exclusively encompassing a single ST, while clade III was more diverse, including isolates belonging to different STs. Isolates recovered from the same herd showed the lowest number of different SNPs, suggesting that the same (or a very similar) ETEC strain circulates in a specific farm.

F4-positive isolates appeared to be more closely related than F18-positive strains, since the maximum number of SNPs detected was lower in this group than among F18 strains (24,593 versus 45,742 SNPs), and the diversity of STs was lower among F4 isolates than F18 strains (three versus six). Results obtained from the phylogeny comparison, including isolates from four different countries, suggest that the ETEC ST100 isolates are closely related, since the SNP differences were relatively small among them. Interestingly, a relatively small number of SNPs (130 to 699) was identified between the F4-positive isolates from the 1990s and those recovered during 2018 to 2019, indicating that very similar clones have been circulating for at least 30 years. To confirm this hypothesis, a larger number of F4 strains from previous years (from the 1990s to now) should be analyzed. The clone O6:H16-ST48, which included 11 isolates which were recovered from five different herds and differed by 6 to 633 SNPs, was the second most prevalent among F4-positive isolates. Notably, O6 is one of the most important ETEC serogroups involved in human diarrhea globally, particularly among children under the age of five in developing countries (38), and it is not classically associated with PWD. A recent study based on the genomic characterization of 40 ETEC O6:H16/HNT human isolates collected during 1975 to 2016 showed significant genomic diversity among them, but none was assigned to ST48 (38). The occurrence of ETEC O6:H16 among pig ETEC isolates indicates zoonotic potential; however, the strains did not harbor known fimbrial genes involved in adhesion to the human intestine.

The World Health Organization has defined AMR as a global health issue in both humans and animals and has recommended surveillance for AMR bacteria in food-producing animals, such as pigs, as they represent a possible source and disseminator of AMR to humans (39). ETEC isolates from pigs are not considered zoonotic (the key virulence factors required to cause disease differ between pigs and humans) (40), but treatment with antimicrobials against PWD may select for AMR in commensal intestinal bacteria, and such bacteria may transfer critical resistances to humans via the food chain. To investigate this aspect, we determined, both phenotypically and *in silico*, the AMR levels among the ETEC isolates. The highest rates of AMR were found against aminoglycosides and sulfamethoxazole, the use of which has increased in livestock in Denmark during recent years (41). Resistance to tetracycline, trimethoprim, and ampicillin, which are among the antimicrobials frequently used for treatment of PWD in Denmark, was broadly detected. Notably, levels of tetracycline and ampicillin resistance were similar to those detected in a previous study on pathogenic *E. coli* from pigs in Denmark (42). All of the isolates were susceptible to ciprofloxacin, cefotaxime, ceftiofur, and colistin, which are critically important antimicrobials for human medicine (15), but treatment with other antimicrobial agents may allow their coselection. The absence of these resistances may be linked to the restricted use of these drug classes in the pig industry, where fluoroquinolones, cephalosporins, and colistin all bear a penalty of factor 10 in the herd-level registration scheme of the use of antimicrobials (the yellow-card scheme) in Danish pig production (41). Similar findings were described in a recent study in Denmark, where the highest proportions of AMR among ETEC isolates were found for ampicillin (60.7%), sulfamethoxazole (69.7%), tetracycline (47.2%), and trimethoprim (69.7%), while AMR to ciprofloxacin, ceftiofur, and colistin was not detected (43). MDR in the pig industry has been linked with the wide use of aminoglycosides and beta-lactams in veterinary medicine (44). Here, MDR was detected in 60 isolates (66.7%), far from the 94% detected among ETEC isolates carrying *mcr-1* and causing PWD in Spain (36). This highlights that the *in silico* prediction of AMR genes showed an almost perfect agreement with the phenotypic analysis according to kappa statistical analysis. In addition, genes encoding macrolide (specifically for erythromycin via MdfA) or lincosamide resistance, which were not phenotypically tested, were also predicted. Both drug classes are commonly employed in Denmark and other countries for treatment against *Lawsonia intracellularis*, an intracellular pathogen causing enteric disease in pigs (45–47). The increase in the administration of macrolides during recent years and the steady use of lincosamides in the pig industry in Denmark (41) could have selected for resistance to the drugs in *E. coli*.

Studies of ETEC across several countries worldwide, including old studies from Denmark, describe ETEC F4 as the most common type associated with PWD, followed by F18 (48–52). However, in other countries, such as Poland, Cuba, Japan, and Spain (36, 53–55), the highest prevalence was found for ETEC F18. In a recent study in Denmark, the number of F4 and F18 strains detected was similar (annual report from 2018; https://diagnostik.dtu.dk/raadgivning/aarsrapporter-for-diagnostik_overvaagning/aarsrapporter-svin). As demonstrated in previous studies, the 90 strains under study were all F4ac or F18ac (6, 7). Further, Denmark has had a breeding strategy to reduce the susceptibility of pigs to ETEC F4ac. The strategy consisted of the inactivation (based on one SNP change) of the candidate gene of the F4ac receptor *MUC4* (5, 56); however, according to our results, F4 strains of type ac are still recovered. Since information on the farms is confidential, we acknowledge whether the herds under study have this strategy implemented and/or piglets were vaccinated. Thus, the reason why F4ac is still being detected could be that some of the isolates tested were recovered from herds where the strategy and/or vaccination has not been applied. Some studies also suggest that *MUC13* and not *MUC4* is the most likely gene governing susceptibility to ETEC F4ac, and this might explain why ETEC F4ac is still the predominant causative agent of PWD right after weaning (57).

In two F4 isolates, F4 and F6 fimbrial genes were detected concurrently. ETEC isolates encoding more than one fimbria have been previously described (28, 48, 49, 58, 59), and such strains have been suggested to have a pathogenetic advantage (27). The most prevalent enterotoxin detected in our study was STb (85 isolates), consistent with previous studies performed in other countries (28, 48, 52). All F4-positive isolates carried *astA*, *ltcA*, and *stb* genes, in line with results of previous studies, where the F4 fimbria-encoding gene was strongly associated with *lt* and *stb* (28, 48, 60). However, in our study, only a portion of F18-positive isolates ($n = 8$) carried *astA*, *ltcA*, and *stb* genes, while the remaining strains harbored *ltcA* and/or *stb* and *sta* genes.

Additionally, 16 other virulence genes were predicted among strains, and their distribution was associated with the type of fimbria, suggesting that F4- and F18-positive isolates use different virulent strategies to cause disease. As suggested, ETEC strains harboring additional fimbrial adhesins may exploit alternative pathways for the colonization of the host (17). Overall, the analysis showed that ETEC isolates from Danish pigs harbor other virulence factors than their characteristic adhesins and toxins, but currently the role for such factors, if any, in intestinal disease is not known.

Plasmids play an important role in the spread and dissemination of both AMR and virulence genes (61). IncF, IncI, and IncX replicon types were the most prevalent, and at least one IncF replicon was detected in all strains, as previously reported in porcine ETEC isolates from Australia and Spain (32, 62). In general, F4 isolates showed more plasmid replicon diversity (21 different plasmid replicons) than the F18 isolates (eight different plasmid replicons). IncF plasmid type is the most commonly described in bacteria from humans and animals, particularly in *E. coli*, and is known to carry virulence and AMR genes (63, 64). IncI1 plasmids, the most dominant among F18 strains, were also detected in all O141-F18 isolates and in 87.1% of O149:F4 isolates from Australia (32). IncI1-type plasmids are often associated with AMR and known to be ESBL carriers (65); however, in the current study, ESBL were not detected, and the link to AMR was not investigated. IncX plasmids are narrow-host-range plasmids of *Enterobacteriaceae*, which are known to provide additional advantages commonly associated with AMR and biofilm formation (64, 66). Since ETEC fimbria and toxin genes often have been reported to be plasmid located (67), we investigated the putative plasmid localization of these genes and potential cooccurrence with AMR determinants. F4 and F18 fimbria-encoding genes were found to be plasmid located in all strains, and toxins were plasmid located in more than 70% of the isolates. In addition, the majority of the AMR genes were predicted to be plasmid located. Interestingly, AMR and virulence genes were often predicted to be part of the same plasmid component; however, further detailed studies are needed to confirm this, since the program used for predictions (plasmidSPAdes) does not separate plasmids of the same type present in a single strain.

Recent studies have reported that AMR might be associated with tolerance to heavy metals existing naturally or used in food animal production, such as zinc oxide and copper (41, 68–70). Here, we predicted that aminoglycoside, tetracycline, and ampicillin resistance genes and zinc or copper tolerance genes were located on the same plasmid component; however, as mentioned above for AMR and virulence genes, more detailed studies are needed to confirm this. Colocalization implies that the use of ZnO and Cu coselects for AMR. In addition to heavy metals, biocides, including disinfectants and antiseptics, widely used in farms, also could promote the spread of AMR (68). All isolates analyzed here were predicted to carry genes responsible for biocidal resistance, but cooccurrence with AMR was not investigated.

Each of the 90 isolates under study contained at least seven prophages, mostly similar to the coliphages P88 (from 3.9% to 93.3%) and Phi27 (from 4.8% to 60.9%). Although Phi27 is known to encode Stx2e, none of the ETEC isolates encoded Shiga toxins, indicating that, despite the similarity, it is not the same phage.

In conclusion, the current study showed a high clonal diversity among F18 isolates, while, in contrast, similar F4 clonal groups might be circulating in Danish herds. High rates of AMR against aminoglycosides, sulfamethoxazole, tetracycline, trimethoprim, and ampicillin were detected, as was the high diversity of virulence genes, including toxin genes (*ltcA*, *astA*, *sta*, and *stb*) and fimbria-encoding genes typed as F4ac and F18ac.

MATERIALS AND METHODS

Bacterial strains and PCR detection of fimbria types F4 and F18. ETEC F4 isolates were collected from pigs with diarrhea in 2018 ($n = 34$, from 30 different farms), 2019 ($n = 29$, from 29 different farms), and 1989 to 1992 ($n = 6$, from six different farms). Presumptive ETEC F18 isolates ($n = 20$) were recovered from five different farms (collected at the same time point on each farm) in 2019. In addition, *E. coli* Nysø, a well-characterized ETEC strain recovered in the 1970s (71), was included as an F18 historical control. Strains were obtained during routine diagnostic procedures, and use for research purposes did not require ethical clearance as long as farm identity was not disclosed.

Strains were confirmed positive for F4 or F18 fimbriae using PCR with primers and conditions as previously reported (52).

Antimicrobial resistance phenotype. MIC values for *E. coli* isolates were determined for amoxicillin (2 to 32 μg/ml)-clavulanic acid (1 to 16 μg/ml), ampicillin (1 to 32 μg/ml), apramycin (4 to 32 μg/ml), cefotaxime (0.125 to 4 μg/ml), ceftiofur (0.5 to 8 μg/ml), chloramphenicol (2 to 64 μg/ml), ciprofloxacin (0.015 to 4 μg/ml), colistin (1 to 16 μg/ml), florfenicol (2 to 64 μg/ml), gentamicin (0.5 to 16 μg/ml), nalidixic acid (4 to 64 μg/ml), neomycin (2 to 32 μg/ml), spectinomycin (16 to 256 μg/ml), streptomycin (8 to 128 μg/ml), sulfamethoxazole (64 to 1024 μg/ml), tetracycline (2 to 32 μg/ml), and trimethoprim (1 to 32 μg/ml) by the broth microdilution method using Sensititre microtiter trays (DKMVN4, Sensititre system; Thermo Fisher Scientific, West Sussex, United Kingdom). *E. coli* ATCC 25922 was used as a quality control. Results were interpreted according to EUCAST epidemiological cutoff values, EUCAST clinical breakpoints for amoxicillin-clavulanic acid (www.EUCAST.org), and DANMAP for apramycin (72). Isolates were defined as susceptible when classified as wild type and resistant when classified as not wild type. Multidrug-resistant (MDR) strains were those resistant to one agent from three or more different antimicrobial classes (73).

PCR for detection of *sta* and *stb* toxin genes. *E. coli* DNA was extracted from a single overnight-grown colony by the boiling lysis method as reported previously (52), and the PCR amplification of *sta* and *stb* toxin genes was performed using the primers and PCR conditions previously described (52).

DNA extraction and whole-genome sequencing (WGS). DNA was extracted using the Maxwell system (Promega) by following the instructions provided by the Maxwell RSC cultured cell DNA kit (Promega). The quality of the DNA was determined by a NanoDrop-1000 (Thermo Fisher Scientific), and DNA quantification was performed using a double-stranded DNA BR assay kit with a Qubit 2.0 fluorometer (Invitrogen, USA).

The libraries for sequencing were prepared using the Nextera DNA Flex library preparation kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's protocol and sequenced using Illumina NextSeq (Illumina). The paired-end raw reads were assembled using SPAdes Genome Assembler v.3.13.0 (74), and the quality of assembly was evaluated with QUAST v.5.0.2. (75).

Whole-genome characterization. The assembled contigs, with genomic sizes between 5.1 and 5.7 Mbp (mean size, 5.4 Mbp) (see Table S1 in the supplemental material), were analyzed using the bioinformatics tools of the Center for Genomic Epidemiology (CGE) for the presence of antibiotic resistance (ResFinder v.3.2) (76), virulence genes (VirulenceFinder v.2.0) (77), and plasmid replicon types (PlasmidFinder v.2.1) (78), as well as identification of clonotypes (CHTyper v.1.0), sequence types (MLST v.2.0) (79), and serotypes (SerotypeFinder v.2.0) (75). All of the CGE predictions were called using default settings. The identification of antibacterial biocide and metal tolerance genes was assessed using BacMet-Scan v.2.0 (80). The ClermonTyping tool (http://clermontyping.iame-research.center/) and PHASTER webserver (http://phaster.ca/) were used to predict the phylogroups and putative prophage sequences in the bacterial genomes, respectively (81, 82).

***In silico* prediction of localization of antimicrobial resistance, virulence, and metal tolerance genes and potential cooccurrence on plasmids.** The putative localization of AMR and virulence genes (F4, F18, *ltcA*, *astA*, *sta*, and *stb*) was predicted using a combination of plasmidSPAdes v.3.13.0 (83), ResFinder, and VirulenceFinder tools. Briefly, plasmidSPAdes was used to identify contigs most likely belonging to plasmid DNA and to assign them to components. Each component is considered a putative plasmid consisting of one or more contigs. This tool is not able to separate similar plasmids (for example, similar plasmids of the same type present in a single strain); thus, their contigs may be assigned to the same identifier (ID) (same component). ResFinder and VirulenceFinder were used to analyze the presence of AMR and virulence genes in all contigs identified as putative DNA regions of plasmids. The output from both tools provides the contig ID and component on which the specific genes were located. Genes contained in the same contig and/or component were predicted to be plasmid located, while those antimicrobial and/or virulence genes not detected in plasmid DNA contigs were assumed to be chromosome located.

The BacMet database (BacMet-Scan v.2.0.), which includes metal tolerance and biocide resistance genes (metal tolerance genes include those genes that are indirectly related to metals) was used to investigate the presence of these genes in all of the genomes. The genome of the *E. coli* K-12 substrain MG1655 (GenBank accession number NC_000913.3) was also included in the analysis. Plasmid contigs identified and assigned to components with plasmidSPAdes v.3.13.0, as described above, were analyzed with BacMet-Scan v.2.0. Next, the plasmid contigs were manually inspected for the presence of metal tolerance genes (zinc, copper, silver, mercury, and tellurium) previously identified on the genome assembly by using the BacMet database (Table S4). The cooccurrence of AMR genes (detected as indicated above) and the metal tolerance genes on the same plasmid were predicted when they were found to belong to the same contig.

Phylogenetic analysis. Phylogenetic relationships between the isolates were analyzed based on SNP trees constructed using the bioinformatics tool CSI Phylogeny v.1.4 (84), available from CGE. The genome of the *E. coli* K-12 substrain MG1655 was included as a reference strain, and CGE default parameters were used during SNP analysis. The phylogenetic tree was visualized and edited by using the bioinformatics tool iTOL v5 (85).

The ETEC isolates under study also were compared with 20 swine ETEC isolates from three different countries (China, the United States, and Spain), as mentioned above. The accession numbers and MLST types of the 20 ETEC strains used in this phylogenetic analysis are indicated in Table S10.

Statistical analysis. Differences between F4- and F18-positive strains regarding serogroups, antimicrobial resistance, virulence gene content, and plasmid replicons were analyzed using two-tailed Fisher's exact test with GraphPad Prism version 8.3 software (GraphPad Inc.). *P* values of <0.05 were considered statistically significant.

Cohen's kappa statistical analysis was used to analyze the correlation between phenotypic resistance and *in silico* gene predictions using SPSS, version 26 (IBM, USA). Kappa values of ≤ 0 indicate no agreement; 0.01 to 0.20, none to slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and 0.81 to 1.00, almost perfect agreement (86).

Data availability. The draft genome sequences of *E. coli* isolates from pigs in Denmark in this study are available in the European Nucleotide Archive (ENA) under the study accession number PRJEB38608.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.9 MB.

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We declare that we have no competing interests.

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